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(54) Title: NEOMORPHOGENESIS OF CARTILAGE *IN VIVO* FROM CELL CULTURE

(57) Abstract

Methods and artificial matrices for the growth and implantation of cartilaginous structures and surfaces and bone are disclosed. In the preferred embodiments, chondrocytes are grown on biodegradable, biocompatible fibrous polymeric matrices. Optionally, the cells are proliferated *in vitro* until an adequate cell volume and density has developed for the cells to survive and proliferate *in vivo*. One advantage of the matrices is that they can be cast or molded into a desired shape, on an individual basis, so that the final product closely resembles a patient's own ear or nose. Alternatively, flexible matrices can be used which can be manipulated at the time of implantation, as in a joint, followed by remodeling through cell growth and proliferation *in vivo*. The cultured cells can also be maintained on the matrix in a nutrient media for production of bioactive molecules such as angiogenesis inhibiting factor. Examples are provided showing the growth of hyaline cartilage for joint relinings, the growth of elastic cartilage for plastic or reconstructive replacement of cartilage structures, and repair of large bone defects.

+ See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

NEOMORPHOGENESIS OF CARTILAGE *IN VIVO* FROM CELL CULTURE
Background of the Invention

This is a continuation-in-part of U.S.
Serial No. 07/339,155 entitled "Neomorphogenesis of
Cartilage *in vivo* from Cell Culture" filed April
5   17, 1989 by Joseph P. Vacanti, Charles A. Vacanti,
and Robert S. Langer, which is a continuation-in-
part of U.S. Serial No. 07/123,579 entitled
Chimeric Neomorphogenesis of Organs by Controlled
Cellular Implantation Using Artificial Matrices
10   filed November 20, 1987 by Joseph P. Vacanti and
Robert S. Langer, which is a continuation-in-part
of U.S. Serial No. 933,018 entitled "Chimeric
Neomorphogenesis of Organs Using Artificial
Matrices" filed November 20, 1986 by Joseph P.
15   Vacanti and Robert S. Langer.

The United States Government has rights in
this invention by virtue of NIH grant No. 6M 26698.

This is generally in the field of medicine
and cell culture, and in particular in the area of
20   implantable cartilaginous structures formed on
biocompatible artificial matrices.

U.S. Serial No. 07/123,579 entitled
Chimeric Neomorphogenesis of Organs by Controlled
Cellular Implantation Using Artificial Matrices
25   filed November 20, 1987, and U.S. Serial No.
06/933,018 entitled "Chimeric Neomorphogenesis of
Organs Using Artificial Matrices" filed November
20, 1986, by Joseph P. Vacanti and Robert S. Langer
describe a method and means whereby cells having a
30   desired function are grown on polymer scaffolding
using cell culture techniques, followed by transfer
of the cell-polymer scaffold into a patient at a
site appropriate for attachment, growth and
function, after attachment and equilibration, to
35   produce a functional organ equivalent. Success

depends on the ability of the implanted cells to
attach to the surrounding environment and to
stimulate angiogenesis. Nutrients and growth
factors are supplied during cell culture allowing
5   for attachment, survival or growth as needed.

           After the structure is implanted and growth
and vascularization take place, the resulting
organoid is a chimera formed of parenchymal
elements of the donated tissue and vascular and
10   matrix elements of the host. The polymer
scaffolding used for the initial cell culture is
constructed of a material which degrades over time
and is therefore not present in the chimeric organ.
Vascular ingrowth following implantation allows for
15   normal feedback mechanisms controlling the soluble
products of the implanted cells. The preferred
material for forming the matrix or support
structure is a biodegradable artificial polymer,
for example, polyglycolic acid, polyorthoester, or
20   polyanhydride, which is degraded by hydrolysis at a
controlled rate and reabsorbed. These materials
provide the maximum control of degradability,
manageability, size and configuration, although
other materials, including non-biodegradble
25   materials such as teflon can be used. In some
embodiments these materials are overlaid with a
second material such as gelatin or agarose to
enhance cell attachment. The polymer matrix must
be configured to provide both adequate sites for
30   attachment and adequate diffusion of nutrients from
the cell culture to maintain cell viability and
growth until the matrix is implanted and
vascularization has occurred. The preferred
structure for organ construction is a fibrous three
35   dimensional structure formed of polymer fibers
having a high surface area, which results in a

-3-

relatively shallow concentration gradient of
nutrients, wastes, and gases, so as to produce
uniform cell growth and proliferation.

U.S. Serial No. 06/933,018 and U. S. Serial
No. 07/123,579 disclose several examples of the
successful culturing and implantation of
hepatocytes and cells isolated from intestine and
pancreas, with subsequent normal function,
including production and secretion of bioactive
molecules. Examples of such molecules include
growth hormone from pituitary cells, insulin and
glycogen from pancreatic cells, and clotting
factors from liver cells. As described in these
applications, however, there is a need for a
different type of functioning "organ", one which
provides primarily a structural function. Examples
of types of cells which are useful in these
applications include cartilage and bone precurser
cells.

Damage of cartilage produced by disease,
such as arthritis, or trauma is a major cause of
physical deformity and debilitation. In medicine
today, the primary therapy for loss of cartilage is
replacement with a prosthetic material, such as
silicone for cosmetic repairs, or metal alloys for
joint relinement. Placement of prostheses is
commonly associated with significant loss of
underlying tissue and bone without recovery of the
full function allowed by the original cartilage, as
well as the irritating presence of a foreign body.
Other long term problems associated with a
permanent foreign body can include infection,
erosion and instability.

The lack of truly biocompatible, functional
prosthesis can have profound and tragic effects for
those individuals who have lost noses or ears due

-4-

to burns or trauma, such as car accidents or war.
The best surgeons can do for these patients is to
carve a piece of cartilage out of a piece of lower
rib to approximate the necessary contours and
5   insert it into a pocket of skin in the area where
the nose or ear is missing.

          In the past, bone has been replaced using
actual segments of sterilized bone or bone powder
or porous surgical steel seeded with bone cells
10 which were then implanted. An example of a process
using bone powder and a hydrated collagen lattice
is U.S. Patent No. 4,485,097 to Bell. An example
of the implantation of a seeded porous metal
prosthesis is U.S. Patent No. 4,553,272 to Mears.
15 The success of these implants has been limited, in
part because of the non-degradable nature of the
cell supports. Very little has ever been actually
used to replace the cartilage overlaying bone
surfaces. At this time, when cartilage is worn or
20 damaged in a joint, there is no way to replace the
cartilage, although International patent
application WO 89/00413 published 26 January 1989
does describe the use of a glycosaminogycan
prosthetic meniscus for implantation in the knee
25 that allows ingrowth of adjoining tissues. Despite
several preparations being tested to stimulate
growth and repair of the remaining cells, in most
cases repair to injuries is made surgically.
Patients suffering from degeneration of cartilage
30 can only turn to drugs having analgesic or
antiinflammatory properties, or compounds such as
hyaluronic acid, for relief.

          To date, the growth of new cartilage from
either transplantation of autologous or allogeneic
35 cartilage has been largely unsuccessful.
Microscopic islands of cartilage formation have

recently been demonstrated histologically *in vivo* by implanting recombinant bone morphogenic protein, as reported by J.M. Wozney, et al., <u>Science</u>, 242, 1528-1534, (December 16, 1988). Limited success

5 has been achieved in making neocartilage using free autogenous grafts of perichondrial flaps, as described by J. Upton, <u>Plastic and Reconstructive Surgery</u>, 68(2), 166-174, (August 1981). However, there have been no reports of successful growth of

10 cartilage *in vivo* from cell culture.

It is therefore an object of the present invention to provide a method and means for designing, constructing and utilizing artificial matrices as temporary scaffolding for cellular

15 growth and implantation of cartilage.

It is a further object of the invention to provide biodegradable, non-toxic matrices which can be utilized for cell growth, both *in vitro* and *in vivo*, as supports for cartilaginous structures.

20 It is a still further object of the invention to provide biodegradable, non-toxic matrices which can be utilized for cell growth, both *in vitro* and *in vivo*, to replace degenerated hyaline cartilage in joints and other places of

25 surface-to-surface contact, and elastic cartilage for plastic and reconstructive surgery.

It is another object of this invention to provide an *in vitro* system in which cells will retain their normal morphology and cell function

30 for the secretion of bioactive molecules normally produced in the body by those cells.

It is still another object of this invention to provide a method and means for growing new bone form cartilage cells implanted on

35 biodegradable, non-toxic matrices

Summary of the Invention

Methods and artificial matrices for the growth and implantation of cartilaginous structures and surfaces and the production of bioactive molecules manufactured by chondrocytes are disclosed. Also disclosed is a method and matrices for growing new bone from chondrocytes implanted on biodegradable matrices.

In the preferred embodiments, chondrocytes are grown in culture on fibrous matrices formed of biodegradable polymers, non-degradable materials, or a combination of the two. The cells can be cultured *in vitro* until an adequate cell volume and density has developed for the cells to survive and proliferate *in vivo*, or maintained *in vitro* for the purpose of manufacturing bioactive molecules, such as angiogenesis inhibiting factor. Alternatively, when adequate cell numbers for implantation are available, the cells can be attached to the matrix and implanted directly, without proliferation *in vitro*. One advantage of polymeric matrices is that they can be cast or molded into a desired shape on an individual basis, so that the final product closely resembles the patient's own ear or nose. Alternatively, flexible biodegradable matrices can be used which allow for manipulation at the time of implantation, as in a joint, followed by remodeling through cell growth and proliferation *in vivo*.

Examples are provided showing the growth of hyaline cartilage for joint relinings, the growth of elastic cartilage for plastic or reconstructive replacement of cartilage structures, and repair of large bone defects.

-7-

Brief Description of the Drawings

Figures 1a and 1b are photographs of the head of a nude mouse implanted with a biodegradable matrix seeded with chondrocytes (Figure 1a before and after implantation); and after five to six weeks of growth, showing cartilage plates underlying the skin (Figure 1b), demonstrating construction of facial contour plates.

Figure 2 is a phase contrast photomicrograph of bovine chondrocytes attached to two polymer fibers three hours after seeding the fibers.

Figure 3 is a photograph of Hematoxylin and Eosin stained chondrocytes after 10 days in culture.

Figure 4a and 4b are phase micrographs of chondroxytes attached to the polymer fibers after 21 days in culture. Figure 4a is a 4x magnification. Figure 4b is a 20x magnification.

Figure 5 is a photomicrograph of polyglactin 910 fibers shaped and seeded with bovine chondrocytes and cultured in nude mice, after 8, 18, 28, 49 and 81 days.

Figure 6 is a photograph at 4x of Hematoxylin and Eosin stained chondrocytes showing a small island of cartilage at the arrow eight days after implantation.

Figure 7 is a photograph at 20x of the cartilage island of Figure 6.

Figure 8 is a photograph at 20x of Hematoxylin and Eosin stained chondrocytes on a polymeric matrix 28 days after implantation, showing the polymers being absorbed by the surrounding tissue.

-8-

Figures 9a and 9b are photographs of
Hematoxylin and Eosin stained chondrocytes 81 days
after implantation in an animal. Figure 9a is at
20x. Figure 9b is at 4x.

5   Figures 10a and 10b are photographs showing
construction of a knee joint lining in a rabbit:
Figure 10a, suturing in a polyglycolic acid
surgical mesh seeded with chondrocytes overlaid
with surgical mesh not seeded with chondrocytes;

10   and Figure 10b, showing formation of a cartilage
plate after five weeks.

Detailed Description of the Invention

As described in more detail below,
dispersed chondrocytes, fibroblasts, and/or bone

15   precursor cells are isolated and then mixed with a
biocompatible matrix *in vitro*, for subsequent
implantation *in vivo* to form a cartilaginous
structure. In one embodiment, cells are incubated
with the matrix until they attach to the surface

20   and the matrix is then implanted at a surgically
prepared recipient site. Methods for preparing the
site are known to those skilled in the art of
reconstructive and plastic surgery. In another
embodiment, the cells are mixed with the matrix,

25   incubated until the cells attach and proliferate to
a desired volume, and then implanted.

The matrices are formed of a biodegradable,
non-degradable, or combination of biodegradable and
non-degradable materials which have been configured

30   to produce high cell densities by allowing adequate
diffusion of nutrients and waste as well as gas
exchange, while *in vitro* or *in vivo*, prior to
remodeling and integration with host tissue.
Cartilage precurser cells, including chondrocytes,

bone precursor cells, fibroblasts, and others,
differ significantly from some types of cells, such
as hepatocytes, in their requirements for nutrient
and gas exchange. As a result, the matrices can be
5   configured as tighter structures than structures
utilized to implant hepatocytes.

Cartilage is a specialized type of dense
connective tissue consisting of cells embedded in a
matrix. There are several kinds of cartilage.
10   Translucent cartilage having a homogeneous matrix
containing collagenous fibers is found in articular
cartilage, in costal cartilages, in the septum of
the nose, in larynx and trachea. Articular
cartilage is hyaline cartilage covering the
15   articular surfaces of bones. Costal cartilage
connects the true ribs and the sternum. Fibrous
cartilage contains collagen fibers. Yellow
cartilage is a network of elastic fibers holding
cartilage cells which is primarily found in the
20   epiglottis, the external ear, and the auditory
tube. As described below, cartilage implants can
be formed of one or more types of cartilage,
depending primarily on the location of the implant
and the type of cartilage cells seeded onto the
25   matrix.

In the preferred method, polymer fibers are
placed in culture media containing chondrocytes,
where the chondrocytes attach to the fibers in
multiple layers and retain their normal rounded
30   configuration, which appears to be essential for
the chondrocytes to maintain their normal function
and secrete a cartilage matrix and other bioactive
molecules such as angiogenesis inhibiting factor.
This technique also allows transplantation of the
35   polymer cell scaffold into animals without
disrupting the complex of attached chondrocytes.

-10-

Transplantation of this complex containing a high
density of normally functioning chondrocytes with a
large surface area into an animal allows the cells
to obtain adequate nutrition by diffusion and
5   successful engraftment of functioning chondrocytes
with cartilage formation prior to remodeling and
integration with the host tissue.

The examples below demonstrate that it is
possible to grow in culture on fibers of
10 biodegradable polymers chondrocytes that appear to
be morphologically and functionally normal, and
will proliferate to a cell density sufficient to
allow implantation of the cell polymer scaffold in
animals and successful engraftment with formation
15 of a new tissue equivalent as the polymer resorbs.

In one example, visual and histologic
characterization of this tissue show that it is
hyaline cartilage (based on the presence of type II
collagen rather than type I collagen), very similar
20 to normal human fetal cartilage. In another
example, chondrocytes obtained from biopsy of
elastic cartilage attached to polymer structures
and grew in the approximate dimensions of the
implanted complex. In still another example, a
25 bone defect was repaired by implantation in the
defect of chondrocytes attached to a polymer
structure, which grew into bone. The examples also
demonstrate that the polymer fiber scaffold is
essential in that neither injection of free
30 chondrocytes nor implantation of the polymer fibers
without attached chondrocytes results in cartilage
formation. Associated with the development of this
cartilage formation is a decrease in
neovascularization and fibrous tissue formation,
35 probably reflecting the production of an
angiogenesis inhibiting factor by the newly formed

-11-

cartilage, as has been demonstrated by assays of
serum in which chondrocytes have been grown *in
vitro* on fibers.

Chondrocytes are initially isolated and
5   cultured using techniques known to those skilled in
the art of tissue culture. In contrast to some
types of cells, chondrocytes can be seeded directly
onto an appropriate matrix and implanted without
first proliferating the cells *in vitro*. If
10   insufficient cell numbers are available for
implantation, cells are first cultured *in vitro* on
the matrix. Once the cells have begun to grow and
cover the matrix, they are implanted in a patient
at a site appropriate for attachment, growth and
15   function. One of the advantages of a biodegradable
polymeric matrix is that angiogenic and other
bioactive compounds can be incorporated directly
into the matrix so that they are slowly released as
the matrix degrades *in vivo*. As the cell-polymer
20   structure is vascularized and the structure
degrades, the cells will differentiate according to
their inherent characteristics.

In the preferred embodiment, the matrix is
formed of a bioabsorbable, or biodegradable,
25   synthetic polymer such as a polyanhydride,
polyorthoester, polyglycolic acid, copolymers,
blends, and combinations thereof. Collagen and
crosslinked glycosaminoglycans can also be used as
the support material. In some cases, non-
30   biodegradable materials such as teflon, nylon,
polyester, or ethylene vinyl acetate can also be
used, alone, or in combination with biodegradable
materials. Although not preferred in the
replacement of linings of joints having surface-to-
35   surface contact, the non-degradable materials have

-12-

structural advantages in some cases, as in the
formation of ears and noses.

Attachment of the cells to the polymer can
be enhanced by coating the polymers with compounds
5   such as basement membrane components, agar,
agarose, gelatin, gum arabic, collagens,
fibronectin, laminin, glycosaminoglycans,
attachment peptides, mixtures thereof, and other
materials having properties similar to biological
10   matrix molecules known to those skilled in the art
of cell culture. All polymers must meet the
mechanical and biochemical parameters necessary to
provide adequate support for the cells with
subsequent growth and proliferation. Factors,
15   including nutrients, growth factors, inducers of
differentiation or de-differentiation, products of
secretion, immunomodulators, inhibitors of
inflammation, regression factors, biologically
active compounds which enhance or allow ingrowth of
20   the lymphatic network or nerve fibers, hyaluronic
acid, and drugs, can be incorporated into the
matrix or provided in conjunction with the matrix.
Similarly, polymers containing peptides such as the
attachment peptide RGD (Arg-Gly-Asp) can be
25   synthesized for use in forming matrices.

A presently preferred polymer is
polyglactin 910, developed as absorbable synthetic
suture material, a 90:10 copolymer of glycolide and
lactide, manufactured as Vicryl[R] braided absorbable
30   suture (Ethicon, Inc., Somerville, New Jersey)
(Craig P.H., Williams J.A., Davis K.W., et al.: A
Biological Comparison of Polyglactin 910 and
Polyglycolic Acid Synthetic Absorbable Sutures.
Surg. 141; 1010, (1975)). A commercially available
35   surgical mesh formed of polyglycolic acid, Dexon[TM],

is preferred for use in construction of new joint
linings.

The polymers can be characterized with
respect to mechanical properties such as tensile
5   strength using an Instron tester, for polymer
molecular weight by gel permeation chromatography
(GPC), glass transition temperature by differential
scanning calorimetry (DSC) and bond structure by
infrared (IR) spectroscopy; with respect to
10   toxicology by initial screening tests involving
Ames assays and teratogenicity assays, and
implantation studies in animals for immunogenicity,
inflammation, release and degradation studies. *In
vitro* cell attachment and viability can be assessed
15   using scanning electron microscopy, histology, and
quantitative assessment with radioisotopes.

Fibers (sutures or non-woven meshes) can be
used as supplied by the manufacturer. Other
shapes can be fabricated using one of the following
20   methods:

Solvent Casting. A solution of polymer in
an appropriate solvent, such as methylene chloride,
is cast on a fibrous pattern relief structure.
After solvent evaporation, a thin film is obtained.
25   Compression Molding. Polymer is pressed
(30,000 psi) into an appropriate pattern.

Filament Drawing. Filaments are drawn from
the molten polymer.

Meshing. A mesh is formed by compressing
30   fibers into a felt-like material.

At the present time, a mesh-like structure
formed of fibers which may be round, scalloped,
flattened, star shaped, solitary or entwined with
other fibers is preferred. The use of fibers is
35   based upon the same principles which nature has
used to solve the problem of increasing surface

-14-

area proportionate to volume increases. All
multicellular organisms utilize a repeating
branching structure. Branching systems represent
communication networks between organs as well as
5   the functional units of individual organs. Seeding
and implanting this type of configuration with
cells allows implantation of large numbers of
cells, each of which is exposed to the environment
of the host, providing for free exchange of
10   nutrients and waste while neovascularization is
achieved.

The polymeric matrix may be made flexible
or rigid, depending on the desired final form,
structure and function. For purposes of making a
15   nose or ear, fibers or sheets of felt-like or solid
material are cut to approximate the plates of
cartilage. Either woven, non-woven or knitted
material can be used. A material such as a velour
is an example of a suitable woven material. The
20   fibers can be fused together by addition of a
solvent or melting to form a more stable structure.
Alternatively, high pressure jets of water onto a
fibrous mat can be used to entangle the fibers to
form a more rigid structure. For resurfacing a
25   joint, a more flexible fibrous mat is cut to
approximate the entire joint surface, then fitted
to the surgically prepared recipient joint as
necessary during implantation. In the preferred
embodiment, the joint is cleaned of existing
30   cartilage, mesh seeded with chondrocytes is used to
line the joint, then mesh not seeded with
chondrocytes is sutured over the seeded mesh, to
secure and protect the chondrocytes until the cells
proliferate, remodel and the resulting cartilage
35   structure is integrated with the surrounding host
tissue and bone. An apparent advantage of using

-15-

the fibrous matrices is the ease in reshaping and rearranging the structures at the time of implantation.

A sponge-like structure can also be used.
5   The structure should be an open cell sponge, one containing voids interconnected with the surface of the structure, to allow adequate surfaces of attachment for sufficient cells to form a viable, functional implant.

10   An advantage of the present invention is that, using similar technology, other components of the nose, ear and joints can be constructed using bone and nerve precurser cells. For example, matrices in the shape of the bones of the inner ear
15 can be formed by casting the polymer to form hollow shapes of the appropriate size and configuration, then seeding with bone precurser cells, culturing *in vitro* as required, then implanting into an ear canal. The major portion of the eustachian tube
20 and inner ear is made of cartilage. The technology is equally applicable to manufacture of an ear drum or skin for covering the implanted cartilage. Nerve cells can also be implanted within or in juxtaposition with the reconstructed ear.

25   The matrix is sterilized prior to mixing with the cells using methods known to those skilled in the art for the matrix material. Materials such as the biodegrables polymers are sterilized with ethylene oxide, carefully removed by degassing
30 prior to mixing the matrix with the cells. Materials such as teflon or nylon can be autoclaved.

Cells may be derived from the host, a related donor or from established cell lines. In
35 one variation of the method using a single matrix for attachment of one or more cell lines, the

-16-

scaffolding is constructed such that initial cell
attachment and growth occur separately within the
matrix for each population, for example, bone
precurser and chondrocyte cell populations.
5   Alternatively, a unitary scaffolding may be formed
of different materials to optimize attachment of
various types of cells at specific locations.
Attachment is a function of both the type of cell
and matrix composition. Chondrocytes obtained from
10   biopsy may be either elastic cartilage or hyaline
cartilage.

These cell-matrix structures are useful not
only for _in vivo_ implantation, but also for the
production of bioactive molecules _in vitro_, such as
15   the proteinase inhibitors reported by Bunning, et
al., _Wur. J. Biochem._ 139, 75-80 (1984) and
Roughley, et al., _Biochem. J._ 169, 721-724 (1978),
and collagenase inhibitor reported by Langer, et
al., _Science_ 191, 70-72 (1976).
20   The following non-limiting examples
demonstrate actual attachment of cell preparations
to bioerodible artificial polymers in cell culture
and implantation and engraftment of this polymer-
cell scaffold into animals. The examples further
25   demonstrate that the cells attached to the matrix
function normally and secrete bioactive molecules,
such as angiogenesis inhibiting factor, and can
therefore be used for the _in vitro_ production of
such molecules.
30   **Example 1:**   **Demonstration of the method for
                      production of cartilage structures** _in_
                      vivo **from polymeric matrices prepared**
                      in vitro.

Figures 1a and 1b demonstrate the method of
35   the present invention for production _in vivo_ of
facial contour plates. Chondrocytes were isolated
from cartilage and and a dispersion prepared using

standard techniques similar to those discussed below. Surgical mesh (Dexon™, a fibrous polyglycolic acid mesh) was seeded with the chondrocytes and the cells allowed to incubate

5 until the chondrocytes attached. The matrix was then implanted on the head of a nude mouse, as shown in Figure 1a. Five to six weeks later, cartilage plates had formed, as shown in Figure 1b.

Example 2: Isolation of chondrocytes from cartilage and analysis of cartilage
10 **formation on a seeded matrix *in vitro* and *in vivo* over time, and lack of formation of cartilage in the absence of a matrix.**

15   Articulating cartilage was obtained from the shoulders of calves under two weeks of age slaughtered earlier in the day. The shoulders were washed in Povidone-Iodine 10% solution (Betadine, Purdue Frederick Co., Norwalk, Conn.), then, under

20 sterile conditions, the muscle attachments were sharply dissected from the underlying bone to expose the joint surfaces. The cartilage from the articulating surfaces of the joint were then sharply dissected from the underlying bone using a

25 #10 scalpel (Bard-Parker, Rutherford, New Jersey). The cartilage was cut into pieces with dimensions of less than 5 mm per side and washed twice in Phosphate Buffered Saline (PBS) with electrolytes and adjusted to neutral pH. The cartilage was then

30 incubated at 37°C in a solution of 0.2% clostridial collagenase (Worthington CLS II, 140 U/mg) and agitated overnight as described by Klagsbrun, (Methods in Enzymology, Vol. VIII). This suspension was then filtered using a 153 μg nylon

35 sieve (Tetko, Elmford, N.Y. 10523). The cells were then removed from the suspension using centrifugation, washed twice with PBS solution and

-18-

counted with a hemocytometer. The solution was
centrifuged at 1800 rpm and the supernatant above
the cell suspension was removed via suction using a
micropipette until the volume of the solution
5   yielded a chondrocyte concentration of 5×10^7
cells/cc.

Braided threads of polyglactin 910, a 90-10
copolymer of glycolide and lactide, coated with
polyglactin 370 and calcium stearate ("0" Vicryl™
10   suture material, Ethicon, Inc., Somerville, New
Jersey) were then cut into pieces of approximately
17 mm in length. One end was unbraided to expose
multiple fibers, 14 microns in diameter. A knot
was placed at the other end to aid in locating the
15   polymer during subsequent biopsy. Two polymer
fibers were placed into each of 26 Falcon tissue
culture dishes, 35 mm in size. Two hundred μL of
the above solution was placed on the two fibers in
each of 15 wells, thus exposing 30 fibers to the
20   solution containing chondrocytes (the
experimentals) and keeping 22 polymers free from
exposure to chondrocytes (the controls). Next, 2
cc of a solution containing Hamm's F-12 culture
media and 10% fetal calf serum with L-glutamine
25   (292 μg/cc), penicillin (100 U/cc), streptomycin
(100 μg/cc) and ascorbic acid (5 μg/cc) was added
to each well. After being incubated at 37°C for 3,
6, 11, 18, 21 and 28 days, six fibers from each
group were examined for the presence and
30   morphologic appearance of chondrocytes using phase
contrast microscopy and then evaluated
histologically using Hematoxylin and Eosin staining
and Aldehyde-Alcian Fuschin stain for chondroitin
sulfate, the strongly acidic sulfate of
35   mucopolysaccharides of the cartilage.

Figure 2 is a phase contrast
photomicrograph of bovine chondrocytes attached to
two polymer fibers three hours after seeding the
fibers. It is important to note that the
5   chondrocytes appear round, their normal
configuration. This configuration is necessary for
secretion of the components of cartilage. Figure 3
is a photograph of the cells stained with
Hematoxylin and Eosin after 10 days in culture.
10 Figure 4a and 4b are phase micrographs of the cells
attached to the polymer fibers after 21 days in
culture. It appears that the chondrocytes cause
the fibers to bend on themselves and come into
contact with other chondrocytes. Figure 4a is a 4x
15 magnification showing the very high density of
chondrocytes filling in the spaces between the
polymer fibers. Figure 4b is a 20x magnification
showing that when the chondrocytes come to the end
of a polymer fiber they seem to multiply to a
20 certain density and form what looks like a node.
After 24 days in culture, the matrix between these
cells stained basophilic by hematoxylin Eosin
staining, demonstrating the presence of cartilage.
The cells are further spaced apart after 24 days in
25 culture than after 10 days. Phase microscopy of
cells after four weeks in culture shows the
chondrocytes bridging the distances between the
polymer fibers.

The remaining forty fibers (24 experimental
30 and 16 control) were surgically implanted
subcutaneously on the dorsum of 40 male nude mice
(Athymic NCr/nude/Sde, Dept. of Radiation Medicine
Massachusetts General Hospital, Boston, MA), four
to five weeks of age, in the midline at the base of
35 the neck. Thirty-five of these implants (19
experimentals and 16 controls) were done after the

-20-

fibers had been incubated for three days *in vitro*,
while the remaining five implants, all
experimentals, were done after incubating the
fibers for 10 days *in vitro*. Five mice with
5   implants (one control, one with chondrocytes
incubated for 10 days and three with chondrocytes
incubated for three days) were sacrificed at each
of the following intervals: 8, 18, 28, 49 and 81
days. The implants were then excised from the
10 surrounding tissue with sharp dissection utilizing
a tissue plane which easily separated the implant
from the surrounding tissue. The specimens thus
included primarily implanted tissue and minimal
endogenous tissue from the animal.
15   Each specimen was fixed in formalin,
weighed, and its volume calculated by measuring the
volume of a liquid which it displaced. Their
weights and volumes were correlated and plotted
against time. All specimens were evaluated grossly
20 and histologically, using Hematoxylin and Eosin
stains as well as an Aldehyde-Alcian Fuschin stain
for the presence of chondroitin sulfate, a major
component of cartilage.
             Figure 5 is a photomicrograph of
25 polyglactin 910 fibers seeded with bovine
chondrocytes and cultured in nude mice, after 8,
18, 28, 49 and 81 days. Figure 6 is a 4x
photograph of Hematoxylin and Eosin stained cells
after 8 days implantation showing a small island of
30 cartilage at the arrow. Figure 7 is a photograph
at 20x of the cartilage island of Figure 6. Figure
8 is a photograph at 20x of an implant after 28
days, showing the polymers being absorbed by the
surrounding tissue. Figure 9a is a photograph at
35 20x after implantation in an animal for 81 days.
Figure 9b is the same implant at 4x, looking very

similar to normal human fetal cartilage at 10
weeks.

In a control study, ten mice were injected
subcutaneously in the same region with a 200 μL

5   suspension containing 5 x 10^5 chondrocytes, without
attachment to polymers. Five of these suspensions
contained chondrocytes isolated primarily from the
calf shoulder and then injected into the mice. The
other five suspensions contained chondrocytes

10   obtained at the same isolation and then incubated
in vitro for three days prior to injection. These
mice were sacrificed at similar time intervals, and
the areas injected were evaluated histologically in
the same manner for evidence of chondrocytes or

15   cartilage.

The results demonstrate that chondrocytes
attach to synthetic biodegradable polymer fibers in
cell culture and proliferate to a cell density
sufficient to allow implantation of the cell

20   polymer scaffold in animals with successful
engraftment and cartilage formation. Fibers of
polyglactin 910 incubated in culture media in the
presence of bovine chondrocytes had chondrocytes
adhering to them and were surgically implanted

25   subcutaneously on the dorsum of twenty nude mice.
As controls, sixteen sets of fibers, incubated in
media not containing chondrocytes, were implanted
in the same manner into sixteen nude mice and ten
mice were injected with 0.2 cc of culture media

30   containing 5 x 10^5 chondrocytes in the same area.

The three groups of mice were sacrificed at
8, 18, 28, 49 and 81 days and the implants were
evaluated grossly and histologically. In eighteen
of the twenty implants with chondrocytes adhering

35   *in vitro*, there was histologic evidence of
cartilage which progressed over the time course of

this study and was indistinguishable in appearance
to normal human fetal cartilage. Furthermore, over
the time course of this study, the polymer fibers
dissolved, beginning by day 27, and, utilizing
5   Hematoxylin and Eosin staining, as well as
Aldehyde-Alcian Fuschin stains, the cartilage
progressed histologically from being isolated
islands of cartilage in the presence of fibrous
tissue and neovascularization at day 8, to becoming
10 a homogenous mass of cartilage. Neovascularization
of the implant with mild inflammation was seen
initially, but over time, the new blood vessels
regressed as cartilage matrix was laid down and
intercellular chondrocyte distances increased as
15 they do with normal cartilage maturation. The
decrease in inflammatory response, as evidenced by
decreases in the number of polymorphonuclear
leukocytes and giant cells, correlated with the
disappearance of the polymers. There was very
20 little evidence of either inflammatory response or
polymer remnants by day 81.

There was no evidence of cartilage present
in any of the control polymeric implants, as
determined histologically using Hematoxylin and
25 Eosin stain. A mild inflammatory response with
polymorphonuclear leukocytes, giant cells, and
fibroblasts was noted until day 28, after which
there was no evidence of the implant. Cartilage
formation was also not evident in any area injected
30 with chondrocytes in suspension.

In conclusion, the chondrocytes readily
adhere to the polymer fibers. The six experimental
fibers incubated in vitro with chondrocytes were
seen microscopically to have multiple layers of
35 chondrocytes adhering to them sufficiently well
that gentle agitation of the complex did not lead

to dissociation of the cells from the fibers. The
cells appeared to remain in their normal rounded
configuration and analysis of the media in which
they were grown demonstrated that angiogenesis
5   inhibiting factor was produced by these cells. The
number of chondrocytes as well as the number of
layers of chondrocytes adhering to the polymer
fibers appeared to increase progressively with time
and appeared to actively remodel the fiber
10   configuration and bridge small distances between
the fibers. The six control fibers incubated in
vitro without chondrocytes showed no evidence of
chondrocytes on histologic evaluation. In vitro,
all polymer fibers (controls and experimentals)
15   began to dissolve by day 27. On gross and
histologic examination using Hematoxylin and Eosin
stain, none of the 16 specimens designated as
controls displayed any evidence of cartilage. In
contrast, 18 of 20 specimens in the experimental
20   group showed evidence of cartilage formation
grossly, as well as histologically using
Hematoxylin and Eosin stain. Histologic
examination of the implants removed at day 8 showed
the fibers were imbedded in fibrous tissue with
25   evidence of a mild inflammatory response consisting
of infiltrates of polymorphonuclear leukocytes and
giant cells, and isolated "nests" of cartilage.
During the time intervals to day 18 and day 28,
these islands of cartilage grew and coalesced into
30   a large homogenous mass of cartilage. There was no
evidence of neovascularization in the 49- and 81-
day implants, and there was decreasing evidence of
an inflammatory response with time as characterized
by a decrease in the number of polymorphonuclear
35   leukocytes and giant cells. Very little evidence
of the polymer fibers was seen after 28 days. This

-24-

increase in the size of the cartilage appeared to
be at the expense of the fibrous tissue previously
seen and associated at least temporarily with a
decrease in neovascularization and resolution of
5   the mild inflammatory response originally noted.
Also associated with this was the absorption of the
biodegradable polymer fibers. In time, the polymer
fibers were progressively replaced by cartilage,
until only cartilage with very little evidence of
10   polymer remained and the specimens became a
homogeneous mass of cartilage histologically very
similar to normal human fetal cartilage. There was
a very positive correlation between the weights and
volumes in both groups, with a rapid decline in the
15   weights and volumes of the control implants with
time after an initial increase in size. The
weights and volumes of the experimentals (those
polymers with attached chondrocytes) initially
paralleled the increase seen in the controls, but
20   then seemed to level off at a stable size by day
49. In the second group of controls, the
injections of free chondrocytes in suspension,
there was no evidence of cartilage formation in any
of the areas injected.

25   **Example 3:   Method of implantation of matrices
                  seeded with chondrocytes and Growth of
                  hyaline cartilage for joint relinings.**

The distal femur of a New Zealand white
rabbit was relined with new hyaline cartilage. As
30   shown in Figure 10a, a surgical Dexon™ mesh seeded
with chondrocytes attached in culture was implanted
into the surgically prepared knee joint of the
rabbit, then overlaid with mesh not seeded with
chondrocytes. The knee was prepared by grinding or
35   slicing off all of the existing cartilage. The
overlaying mesh is sutured over the seeded mesh to

secure and protect the chondrocytes until they proliferate and form a new lining, as shown five weeks after implantation, in Figure 10b.

5   The cartilage appears, both grossly and histologically, to adhere to the underlying bone in a manner consistant with a normal cartilage-bone interface. Analysis of the newly grown cartilage via immunochemistry using the avid-biotin-peroxidase complex technique has demonstrated the

10 presence of type II collagen, and the absence of type I collagen. This is significant in that type II collagen is found almost exclusively in hyaline cartilage, while type I is absent in normal hyaline cartilage.

15   The number of cells needed for implantation to optimally repair a defect in hyaline articular cartilage. The calculations are based on known concentrations of chondrocytes per unit mass of hyaline articular cartilage and the actual counts

20 of the number of chondrocytes present in the cartilage removed when creating a defect of known size. If necessary, the chondrocytes obtained from biopsy can be multiplied *in vitro* prior to attaching the optimal number of cells to the

25 polymers after implantation.

Example 4:   Growth of normal elastic cartilage *in vivo* from matrices seeded with chondrocytes.

Chondrocytes obtained from biopsy of human

30 elastic cartilage was attached to polymer fibers *in vitro* and implanted. Histologically normal elastic cartilge grew in the approximate dimensions of the implanted complex.

-26-

Example 5:   **Implantation of chondrocytes on a**
matrix for repair of a bone defect and
subsequent formation of "bone".

A defect in the tibia of a New Zealand

5   white rabbit of approximately twice the radius of

the bone was created. The periosteum was removed

at the site of the defect. Polymer mesh was seeded

with chondrocytes as described above, and implanted

into the defect. Histology and chemical analysis

10   can be used to confirm that the implant formed

bone. In controls using the same model without the

seeded implant, the defect fills with fibrous

tissue rather than bone.

Although this invention has been described

15   with reference to specific embodiments, variations

and modifications of the method and means for

constructing cartilage implants by culturing

chondrocytes on matrices having maximized surface

area and exposure to the surrounding nutrient-

20   containing environment will be apparent to those

skilled in the art. Such modifications and

variations are intended to come within the scope of

the appended claims.

We claim:

1. A system for growing a cartilaginous structure comprising

a biocompatible matrix in a nutrient environment and

chondrocyte cells attached to the matrix,

wherein the matrix is structured to provide free exchange of nutrients and waste to the attached cells in the absence of vascularization.

2. The system of claim 1 wherein the matrix is formed of a biodegradable material selected from the group of materials degrading by hydrolysis and the group of materials degrading enzymatically.

3. The system of claim 2 wherein the material is selected from the group of polymers consisting of polyanhydrides, polyorthoesters, polyglycolic acids, polylactic acids, collagens, copolymers, blends and combinations thereof.

4. The system of claim 1 wherein the material is selected from the group consisting of teflon, nylon, ethylene vinyl acetate, polyesters and combinations thereof.

5. The system of claim 1 wherein the matrix is formed of a combination of biodegradable and non-degradable materials.

6. The system of claim 1 further comprising coatings on the matrix material selected from the group consisting of basement membrane components, agar, agarose, gelatin, gum arabic, collagens, fibronectin, laminin, hyaluronic acid, glycosaminoglycans, attachment peptides and mixtures thereof.

7. The system of claim 1 further comprising cells selected from the group consisting of bone, skin and nerve precurser cells.

8. The system of claim 1 wherein the matrix forms a rigid structure.

9. The system of claim 1 wherein the matrix forms a flexible structure conformable to a joint surface.

10. The system of claim 1 wherein the matrix is formed of fibers or a fibrous mesh.

11. The system of claim 1 wherein bioactive molecules are produced by the cartilaginous structure.

12. The system of claim 11 wherein the bioactive molecules are secreted into the nutrient environment.

13. The system of claim 1 for growing a cartilaginous structure further comprising a substance in the nutrient environment or polymer having an effect on the chondrocyte cells.

14. The system of claim 1 for growing a cartilaginous structure further comprising cells capable of differentiating into cartilage cells.

15. The system of claim 1 for growing a cartilaginous structure wherein the chondrocytes have formed a cartilaginous matrix.

16. A method for making a cartilaginous structure comprising

providing a biocompatible matrix in a nutrient environment and

attaching cartilage cells to the matrix to form a cartilaginous structure.

17. The method of claim 16 wherein the matrix is formed of a biodegradable material selected from the group of materials degrading by hydrolysis and the group of materials degrading enzymatically.

-29-

18.   The method of claim 17 wherein the material is selected from the group of polymers consisting of polyanhydrides, polyorthoesters, polyglycolic acids, polylactic acids, collagens, copolymers, blends and combinations thereof.

19.   The method of claim 16 wherein the material is selected from the group consisting of teflon, nylon, ethylene vinyl acetate, polyesters and combinations thereof.

20.   The method of claim 16 wherein the matrix is formed of a combination of biodegradable and non-degradable materials.

21.   The method of claim 16 further comprising coating the matrix with a material selected from the group consisting of basement membrane components, agar, agarose, gelatin, gum arabic, collagens, fibronectin, laminin, hyaluronic acid, glycosaminoglycans, and mixtures thereof.

22.   The method of claim 16 further comprising providing cells selected from the group consisting of bone, skin and nerve precurser cells.

23.   The method of claim 16 wherein the matrix is formed as a rigid structure.

24.   The method of claim 16 wherein the matrix is formed as a flexible structure conformable to a joint surface.

25.   The method of claim 16 wherein the nutrient environment is *in vivo* further comprising attaching the cells on a matrix and implanting the attached cells on the matrix in an animal without first proliferating the cells on the matrix *in vitro*.

26. The method of claim 16 further comprising attaching the cells on a matrix, proliferating the cells on the matrix *in vitro* in a nutrient media, then implanting the cells on the matrix *in vivo*.

27. The method of claim 16 further comprising implanting the structure as part of a facial appendage.

28. The method of claim 16 further comprising implanting the structure on the surface of a joint.

29. The method of claim 16 for producing bioactive molecules *in vitro* further comprising culturing the cells on the matrix *in vitro* in a nutrient medium until bioactive molecule is produced.

30. The method of claim 29 further comprising extracting the bioactive molecule from the nutrient medium.

31. The method of claim 29 further comprising extracting the bioactive molecule from the cells on the matrix.

32. The method of claim 16 further comprising providing a substance in the nutrient environment or polymer and determining if the substance has an effect on the chondrocyte cells.

33. The method of claim 16 further comprising characterizing the effect of the substance on the chondrocyte cells.

34. The method of claim 16 further comprising providing cells capable of differentiating into cartilage cells and inducing differentiation into cartilage cells.

35. The method of claim 19 wherein the cartilage cells are induced to form bone cells.

36. The method of claim 16 wherein the structure is implanted to repair cartilage damaged by inflammation.

37. The method of claim 16 wherein the structure is implanted to repair cartilage damaged by trauma.

38. The method of claim 16 wherein the structure is implanted to repair cartilage damaged by aging.

39. The method of claim 16 wherein the structure is implanted to repair cartilage when is congenitally defective.
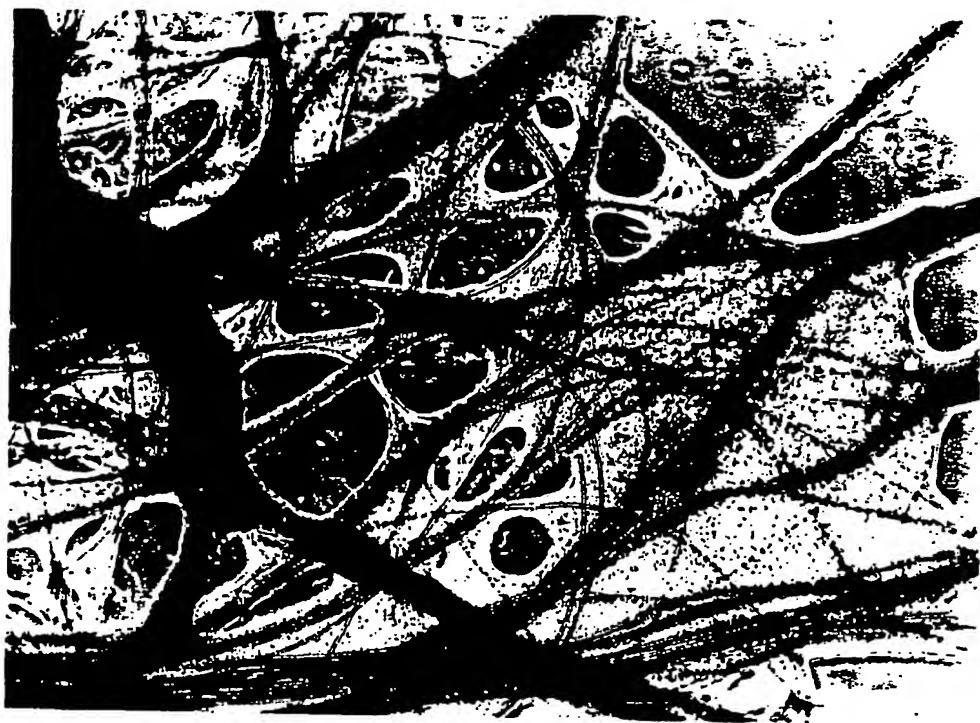
FIG.1A-1
AFTER

FIG.1A-11
BEFORE

FIG.IB

FIG.2

FIG.3

FIG.4A
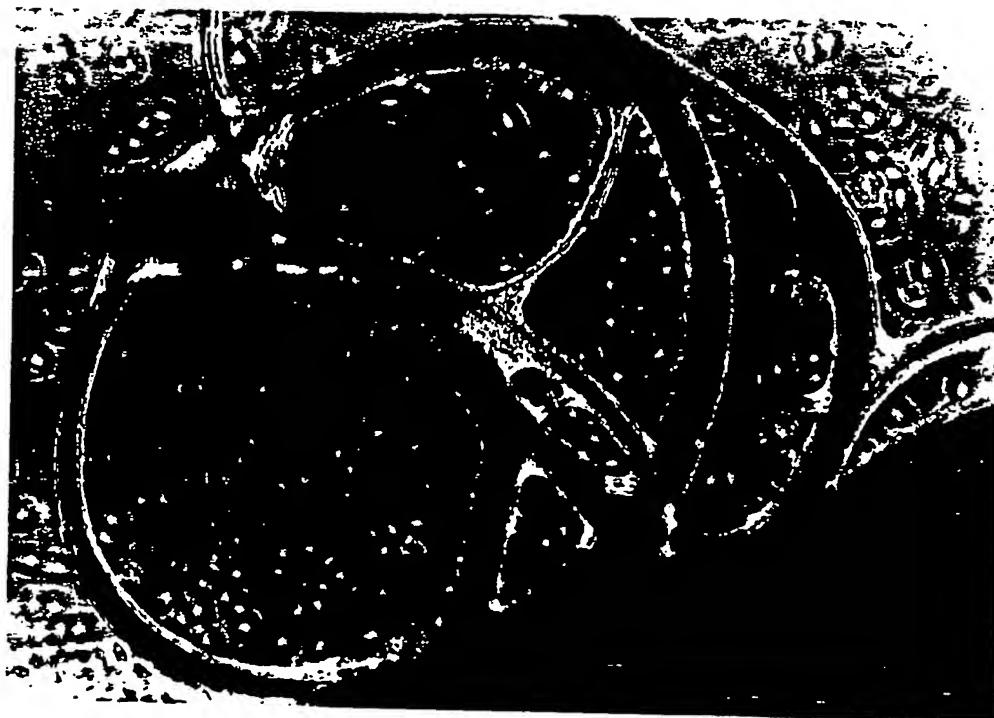

FIG.4B
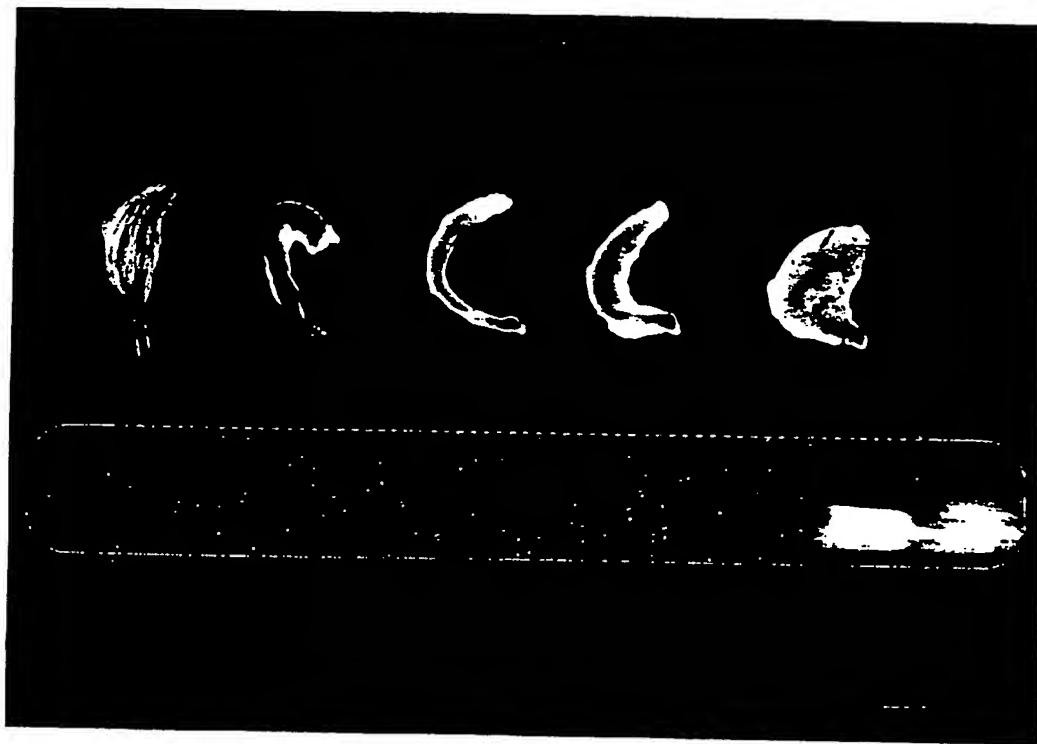
FIG.5

FIG.6
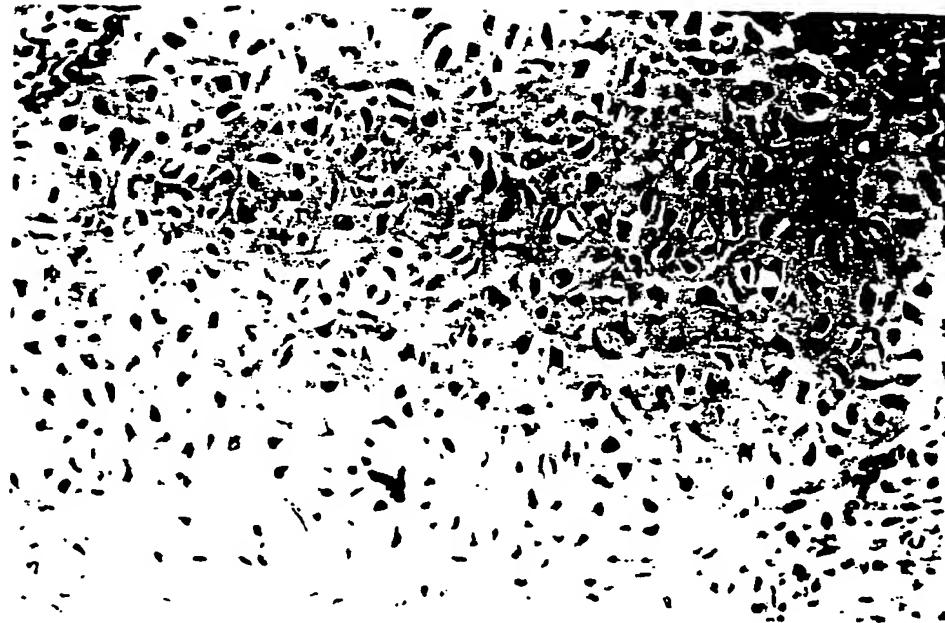
FIG.7

FIG.8

7 / 9
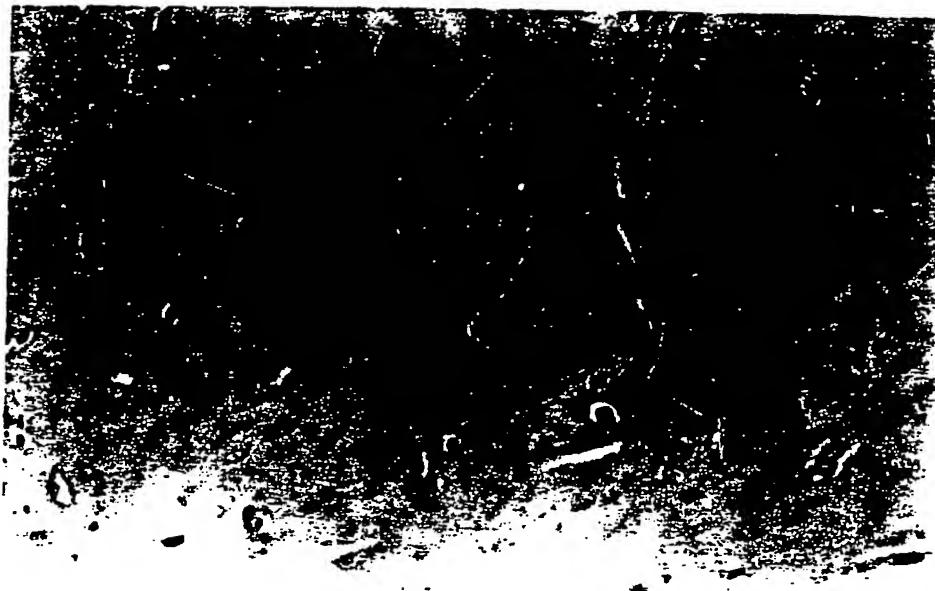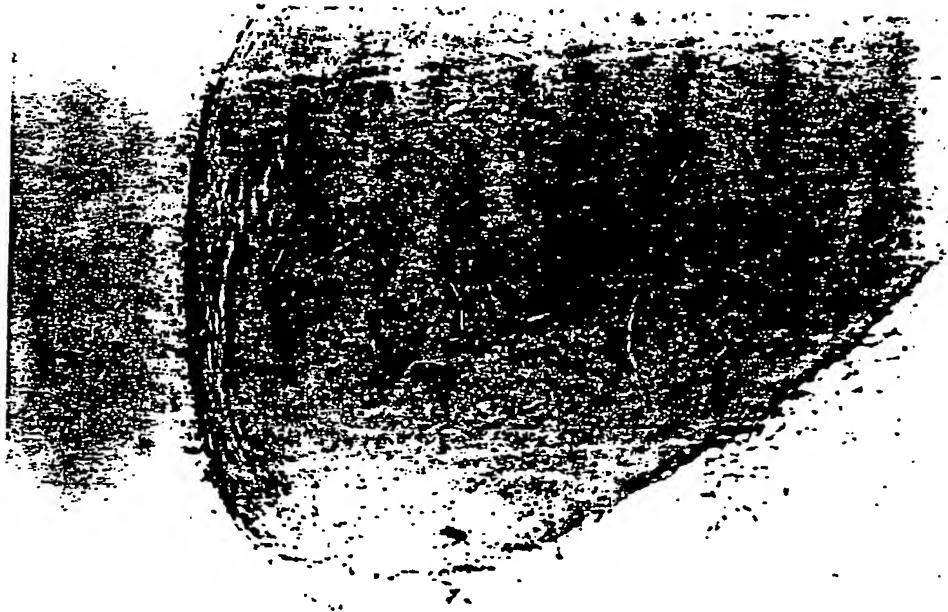

FIG.9A

FIG.9B

FIG.IOA

FIG.IOB

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: A 61 L 27/00,// A 61 F 2/30, C 12 N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC^5	A 61 L

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included In the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	WO, A, 88/03785 (J.P. VACANTI et al.) 2 June 1988 see page 11, lines 16-25; page 12, lines 19-33; page 13, lines 1-2; page 13, lines 21-24 (cited in the application) --	1-3,6-8, 11-18,21-24, 29-35
X	WO, A, 89/00413 (K.R. STONE) 26 January 1989 see claims 1-20 (cited in the application) --	1-3,6-10, 14-18,21-24
P,X	US, A, 4846835 (D.A. GRANDE) 11 July 1989 see column 4, lines 52-57; column 5, lines 3-9; column 9, lines 36-49 --	1-3,9,16-18, 24

./.

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17th July 1990	1 0 AOUT 1990
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	VAN HASS T. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Exp. Eye Res., vol. 24, no. 3, 1977, M. Ptasinska-Urbanska et al.: "Intrascleral introduction of isolated allogeneic chondrocytes capable of cartilage reformation in rabbits; possible procedure in treatment of detachment of the retina", pages 241-247, see pages 242,246	1-3,16-18
	--	
Y	US, A, 4553272 (D.C. MEARS) 19 November 1985 see example 1 (cited in the application)	1-3,16-18
	--	
P,Y	EP, A, 0339607 (S. ITAY, Dr.) 2 November 1989 see examples 1-4	1-3,16-18
	--	
A	EP, A, 0282746 (TAKEDA CHEMICAL INDUSTRIES, LTD) 21 September 1988	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.[X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.[X] Claim numbers __*__, because they relate to subject matter not required to be searched by this Authority, namely:

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* 25-28,36-39
  See PCT-RUle 39.1 (iv): methods for treatment of the human or
                          animal body by surgery or therapy as
                          well as diagnostic methods
```

2.[ ] Claim numbers........., because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3.[ ] Claim numbers........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.[ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple Inventions in this International application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.[ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 9002091
SA   36616

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 02/08/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8803785	02-06-88	EP-A-	0299010	18-01-89
		JP-T-	1501362	18-05-89
WO-A- 8900413	26-01-89	US-A-	4880429	14-11-89
		AU-A-	2424588	13-02-89
		EP-A-	0324852	26-07-89
US-A- 4846835	11-07-89	None		
US-A- 4553272	19-11-85	None		
EP-A- 0339607	02-11-89	US-A-	4904259	27-02-90
		AU-A-	3380889	02-11-89
EP-A- 0282746	21-09-88	JP-A-	1010983	13-01-89

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